Microbial Toxins

Volume III

Bacterial Protein Toxins

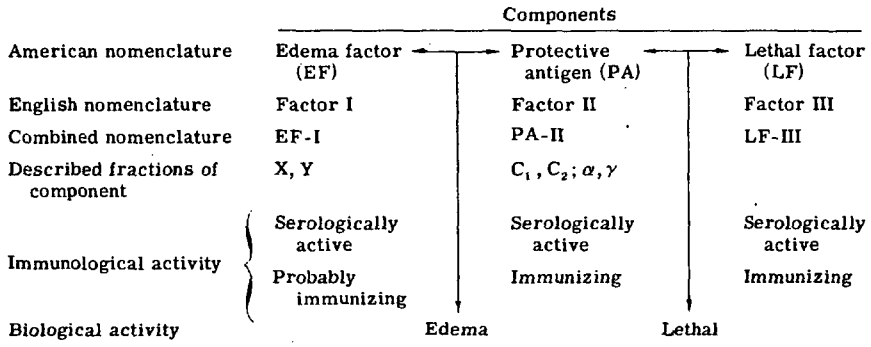
I. Introduction

Anthrax has long been of interest to man. It is believed to have been one of the seven plagues suffered by the Egyptians in the time of Moses and was clearly described in ancient Greece. The disease is of historical importance to all microbiologists: Robert Koch used it as the model for his postulates to prove the bacterial etiology of disease; Pasteur used the causative organism *Bacillus anthracis* to develop an effective attenuated live vaccine.

Although anthrax is one of the five major livestock diseases of the tropics (National Academy of Sciences, 1962), the incidence in man is low and it occurs among veterinarians, meat workers, and, in the more developed countries, workers in woolen and goat hair mills. Anthrax is classified (Cecil and Loeb, 1959) as external (carbuncular) or internal (generalized or septicemic). Carbuncular, the most common type, occurs following skin contact with infected materials. This phase of the disease results in a very characteristic, intensely inflamed, yet painless, carbuncle covered with a black eschar. Internal anthrax occurs on a primary basis following respiratory exposure, gastrointestinal challenge, or wound infection, and on a secondary basis (20%) from untreated carbuncular cases. Generalized anthrax is characterized by an incubation period in which clinical symptoms are either nonexistent or minimal and nonspecific followed by acute respiratory distress, shock, and rapid progress to death. There are few recorded recoveries from internal anthrax because of the nonspecific symptoms and the fact that identification of the disease is based on the presence of gram-positive bacilli in the blood. As a consequence, the literature on treatment of generalized anthrax is very small, and most work has been directed toward the development of a vaccine that will protect against infection.

Only since 1954, when the toxigenic nature of this disease was demonstrated (H. Smith and Keppie, 1954), has any real progress been made in understanding the pathophysiology and the bases for treatment and immunity. Although the Porton group showed the marked correlation in the disease syndrome between its toxigenic and bacillary aspects, no general study of the toxemia was published until our 1968 series (Vick *et al.*,

Fig. 1. Nomenclature and activities associated with toxin components. From Fish and Lincoln (1967).



ity) does not occur unless the various components are combined. PA and LF must be combined before the toxin is lethal, and PA and EF must be combined before the toxin is dermonecrotic. EF and LF combined have no biological activity. The three components combined are immunogenic, lethal, and dermonecrotic. Perhaps the best way to visualize the toxin molecule is in relation to an enzyme. Although all three components appear to be protein, the PA component may be visualized as a coenzyme, while the EF and LF components may be visualized as apoenzymes. There is also some evidence that the toxin can exist in isoenzyme forms.

In this review, when we discuss a particular component as PA, EF, or LF; we will be referring to its individual activity as distinct from both that of any other component or that when combined with another component. Unfortunately, the literature prior to 1961 does not include any mention of LF because that component was not separated from EF until then (Stanley and Smith, 1961; Beall *et al.*, 1962). When referring to these early papers, the reader must remember that EF, factor I, and filter factor all refer to a mixture of EF and LF.

A second area of confusion exists because one of the components (PA or protective antigen) bears the same name as the immunogen called protective antigen. All anthrax immunogens have been called protective antigen without regard for prior usage of the term or proof of identity of the material and, as illustrated in Section VII, have varied from extracts of animal tissue to an alum-precipitated *in vitro* material. Unfortunately, the components present in the various immunogens have not been identified and characterized, and it is impossible to present any reasonable conclusions in this regard here. Consequently, when we use the term "protective antigen," we will be referring to an immunogen of unknown composition. In contrast, when we speak of PA, we will be speaking of one of three readily distinguishable components of the toxin. In order to fully under-

with anthrax infections. These protective and tissue-damaging activities appeared to be localized in sterile extracts from anthrax lesions (Cromartie *et al.*, 1947b). These extracts produced lesions when reinjected into rabbits and continued injection immunized against spore challenge. This diffusible factor or toxin elaborated during growth (Cromartie *et al.*, 1947b) was fractionated into two substances, "one an inflammatory, tissue-damaging factor and the second a protective antigen" (Watson *et al.*, 1947). The protective antigen was shown to be an effective immunogen (Gladstone, 1946; Heckley *et al.*, 1949; Wright *et al.*, 1951), but the relationship between Cromartie's diffusible factor and the present toxin (Fish and Lincoln, 1967) has not been resolved. With the further observations of a capsular and a somatic polypeptide antigen, it is no wonder that the literature has been divided until quite recently into studies on synthesis and production either of toxin (Section III) or of "protective antigen" (Section VII).

II. Quantitation of Toxin

Quantitation of anthrax toxin and comparison of results among the various groups working on the toxin are quite difficult because the toxin may be and has been assayed on the bases of lethality, edema production, protection against challenge, and antigen–antibody interaction (Fish and Lincoln, 1967). The correlation among these different assay procedures is not constant (Puziss and Wright, 1954, 1963; Thorne, 1960; Haines *et al.*, 1965; Fish *et al.*, 1968a). Because the toxin is a complex one, this problem is further complicated by the fact that the assay for one toxin component, although valid, will not always indicate the extent of contamination by a second or third component.

To date, three components have been identified and described in some detail, but more components probably are present (Wright and Luksas, 1964), and those already identified may exist in different ratios or molecular states (Fish and Lincoln, 1967; Fish *et al.*, 1968a; Buzzell, 1967). Disk electrophoresis has shown that crude preparations of anthrax toxin contain six (Baier, personal communication) or sixteen (Wilkie and Ward, 1967) protein bands, so future study probably will reveal interactions of which we know nothing at this time.

Interpretations of biological assays made prior to 1961 must be made in the light of the data indicating that EF (Stanley and Smith, 1961) and "filter factor" (Beall *et al.*, 1962) were in reality combinations of EF and LF. While none of the components alone or EF and LF in combination produce evidence of lethality or edema formation, the individual components are able to affect host physiology (Vick *et al.*, 1968; Fish *et al.*, 1968b).

tion) believe that EF is required for maximum lethality in the mouse if not in all animals. As determined by Ouchterlony assay, the minimal PA:LF ratios that caused death in a rat were 10:8 (Molnar and Altenbern, 1963) and 32:4 or 16:8 (Fish *et al.*, 1968a).

2. Skin Edema Formation

a. Whole toxin. Toxin has been quantitated by either (1) measuring the diameter and depth of the dermonecrotic, edematous lesion produced, or (2) determining the highest dilution of toxin that will produce such a lesion after ID injection in a guinea pig or rabbit (Belton and Henderson, 1956; Thorne *et al.*, 1960; Beall *et al.*, 1962; Fish and Lincoln, 1968). Terminal serum of guinea pigs dying of anthrax produced lesions 40 and 15 mm in diameter when 0.2 ml of undiluted and a 1:8 dilution, respectively, were injected ID in guinea pigs (H. Smith *et al.*, 1955b). Rabbit skin is five- to eight-fold more sensitive to toxin than guinea pig skin (Belton and Henderson, 1956). When assaying toxin produced *in vivo*, it is quite important that the assay be performed soon after obtaining the sample because activity is lost rapidly, even when stored at −20°C (Fish and Lincoln, 1968).

b. Components. Only EF and PA are required to produce a skin edema and lesion corresponding to that observed following injection of the whole toxin. From material produced *in vivo*, the minimal amounts of purified EF-LF mixture and PA required to produce a detectable edema in rabbit skin are 0.3 and 15 μg, respectively (Stanley *et al.*, 1960). Fish *et al.* (1968a) reported a minimum requirement of 18 μg protein (EF purified 35-fold from toxin produced *in vitro*) in combination with PA to yield a visible edema response in guinea pigs.

B. Serological Assay Procedures

1. Ouchterlony

Using the Ouchterlony technique (Ouchterlony, 1953), a concentration as low as 4 μg/ml was measured and correlated with the rabbit skin test and rabbit immunization (Thorne and Belton, 1957). The minimal amounts of each component that have been detected by this procedure are: PA, 0.02 μg (Strange and Thorne, 1958); LF, 0.02 μg (H. Smith and Stanley, 1962); and EF, 0.05 μg (Stanley and Smith, 1961). This assay procedure is the most widely used method for quantitation (Strange and Thorne, 1958; Thorne *et al.*, 1960; Fish and Lincoln, 1967) and has remained essentially unchanged, except for a 3 to 4 fold $\log_2$ increase in titer as a result of equipment modification (Ray and Kadull, 1964) and the use

D. Summary and Future Research

Biological (lethality, skin edema) and serological (Ouchterlony) assays for the toxin and its three known components exist. Standardization is needed among groups working with these procedures and materials. Because each assay measures some different capacity of the toxin, all workers should use several different assays. More also needs to be done to correlate results from one assay with those obtained by another procedure.

A vital need exists for a monovalent antiserum for each component so that (1) an individual line of precipitation on an Ouchterlony plate can be unambiguously identified with a biological function, (2) the complex molecular configuration of the toxin can be interpreted, and (3) the graded responses of the various assay systems can be standardized as the toxin is progressively inactivated. Use of monovalent antiserum, as well as antiserum other than the equine antiserum prepared against spores of the Sterne strain, will aid in studies of possible differences among toxins produced *in vivo* and *in vitro* and by strains of varying degrees of virulence.

III. Production and Purification

This section covers the production and purification of toxin per se, whereas the production of an immunogen is discussed in Section VII.

The elegant and simple experiments of H. Smith and Keppie (1954) clearly identified a toxin which was later shown to be an aggressin (H. Smith and Gallop, 1956) similar to that produced *in vitro* (Gladstone, 1946) or *in vivo* (Cromartie *et al.*, 1947b; Watson *et al.*, 1947). The work of H. Smith and Keppie (1954) stimulated recent work on isolation and purification of toxin, resulting in our advances in (1) understanding the mechanism of action, (2) the development of an effective vaccine, and (3) better methods of treatment. Toxin purification was reviewed by Fish and Lincoln (1967).

A. *In Vivo* Toxin

1. Production

For large-scale production of *in vivo* toxin, H. Smith *et al.* (1953) infected large guinea pigs with spores of the Vollum NP strain of *B. anthracis*. The thoracic and peritoneal exudates, collected immediately at death, were mixed, centrifuged to remove guinea pig and bacterial cells, and sterilized by filtration before purification.

Both components (EF–LF mixture and PA) recognized and isolated by Smith and colleagues appear to be present in the plasma in approximately

et al., 1961) demonstrated that toxin is present in the medium for the short time during which the bacterial concentration is approximately 5×10^7 to 10×10^7 chains/ml. Additionally, certain factors like bicarbonate (Gladstone, 1946; Puziss and Wright, 1954; Harris-Smith *et al.*, 1958; Puziss and Howard, 1963), charcoal (Strange and Thorne, 1958), and sugar source (Puziss and Wright, 1959) are important. Recent work indicates that bicarbonate is required early in the growth cycle and that it (1) affects cell permeability and release of the toxin from the cell to the medium (Puziss and Howard, 1963) and (2) does not merely slow the rate of bacterial growth so that toxin persists in the culture (Harris-Smith *et al.*, 1958).

The early literature on toxin production is clouded by the uncertainty as to whether a high pH and/or the presence of serum was needed for production, separation, or stability of the toxin (Gladstone, 1948; Wright *et al.*, 1954a; Thorne and Belton, 1957; Strange and Thorne, 1958; Harris-Smith *et al.*, 1958; Thorne, 1960; Stanley and Smith, 1961, 1963). Consequently, serum was added before processing (Thorne *et al.*, 1960; Beall *et al.*, 1962; Mahlandt *et al.*, 1966), after processing (Wilkie and Ward, 1967), or not at all (Mahlandt *et al.*, 1966; Fish *et al.*, 1968a). Additional confusion was added by the assay system because the presence of serum or high pH prevented the separation of the components (Beall *et al.*, 1962).

The hypovirulent Sterne strain has been used most often for the production of toxin because it produces slightly higher concentrations of toxin than the NP mutant of the virulent V1-b strain (Harris-Smith *et al.*, 1958). Apparently identical toxin is produced by both nonproteolytic (NP) as well as proteolytic strains of V1-b (Wright *et al.*, 1951; Harris-Smith *et al.*, 1958); thus, proteolytic activity does not appear to be a prerequisite for toxin production.

A reliable and standardized method exists for producing the toxin from the Sterne strain grown on medium 599 with minor modifications (Haines *et al.*, 1965), and all three components have been isolated from this toxin.

2. Purification

Because the toxin is composed of several different components, purification may proceed by (1) concentrating the whole toxin, while keeping the molecular configurations and ratios of all components unchanged or (2) purifying the individual components, and then recombining them to make whole toxin.

a. Whole toxin. Only one group has attempted to purify the whole toxin (Wilkie and Ward, 1967). The toxin, sterilized by Millipore filtration, was concentrated 500- to 1000-fold by ultrafiltration. This resulted in a

contaminated with EF or LF, is serologically distinct from EF or LF, is immunologically active, is dermonecrotic in combination with EF, and, with LF, is lethal.

iii. *Lethal factor component.* LF was purified 3.5-fold with 24% recovery by glass filtration, DEAE-cellulose and hydroxyapatite chromatography, and ammonium sulfate precipitation (H. Smith and Stanley, 1962). The purified component showed a single peak in the ultracentrifuge and by paper electrophoresis; however, under certain conditions, two bands were observed on Ouchterlony gel plates. We (Fish *et al.*, 1968a) purified LF 1025-fold with 11% recovery by glass filtration, Sephadex chromatography, and absorption and elution from calcium phosphate gel. This purified material was serologically distinct from the other two components.

C. Summary and Future Research

The toxin and its three components are produced *in vivo* and accumulate in the terminal serum of infected hosts. Smith and colleagues purified this material about 20-fold with 20% recovery, but they completed this work prior to the identification of LF, so they had only two factors (an EF–LF mixture and PA). They also were unable to remove more than 85% of the guinea pig plasma components from their final preparations. We have shown that the titer and number of lines of precipitation on Ouchterlony plates continue to increase until the death of the guinea pig. Future research, using a kinetic approach as well as different strains, should contribute greatly to studies on the production of this toxin.

Production of toxin or its components is readily accomplished *in vitro* in complex or synthetic media. EF, PA, and LF have been separated and purified 197-, 156-, and 1025-fold, respectively. These isolated components are immunologically homogeneous and distinct, and they remain biologically, immunogenically, and serologically active.

Although methods now exist for the production, isolation, and purification of the three components from the Sterne strain, more needs to be done to ascertain the correct interaction among these components and their spatial configuration or structure. The possibility of new components and a comparison of toxins produced *in vivo* and *in vitro* are two areas in which future research would be most productive.

Whenever the toxin or its components are purified, the properties of the purified materials must be checked against both the original material and toxin produced *in vivo*.

Although existing information indicates that toxin produced by one strain resembles quite closely that of another strain, only toxin produced

at 0°C, thus removing a possible criticism of the temperature stability studies of Fish *et al.* (1968a), who showed that biological activity was lost before serological activity.

As the various components become more purified, they become increasingly labile in the absence of salts (Strange and Thorne, 1958; Stanley *et al.*, 1960; Fish, unpublished observations). Stanley *et al.* (1960) and Stanley and Smith (1961), using toxin produced *in vivo*, reported that crude EF was destroyed by reducing agents but stabilized by mild oxidizing agents, while purified EF was not stable in the presence of either. PA was labile to both oxidizing and reducing agents (Stanley *et al.*, 1960). Fish *et al.* (1968a), using toxin produced *in vitro*, reported that all three components, even when partially purified, appeared to be stable in the presence of oxidizing or reducing agents, but LF and especially PA were quite susceptible to hydrogen bond disrupting reagents.

The pH range for maximum stability of the components is quite narrow, centering at pH 7.4–8.0, and pH becomes more critical as the components are purified (Gladstone, 1946; H. Smith *et al.*, 1955b; Strange and Thorne, 1958; Thorne *et al.*, 1960; Stanley *et al.*, 1960; Stanley and Smith, 1961; Fish *et al.*, 1968a). These results all contradict those of Wright *et al.* (1954b) who report more potent filtrates of "protective antigen" at pH's up to 8.7.

Evidence for molecular heterogeneity (polymerization, aggregation, differential destruction) has been accumulating since H. Smith and Gallop (1956) first observed that "factor X" seemed to be formed by aggregation of "factor Y" as a result of repeated ultracentrifugation. Strange and Thorne (1958) found one peak of PA activity by paper electrophoresis and two peaks by ultracentrifugation. They postulated degradation of the antigen to account for the additional lines appearing on Ouchterlony plates as the culture passed its peak titer and activity was lost. Similar observations were reported by Wilkie and Ward (1967) using disk electrophoresis. Stanley *et al.* (1960) demonstrated that (1) the relative proportion of peaks observed in the ultracentrifuge depended upon the freshness of the toxin, and (2) mild treatment (heating to 37°C) increased the proportion of high molecular weight peaks. On this basis they postulated that EF formed aggregates rather than dissociating to form smaller components. Sargeant *et al.* (1960) reported that EF and PA combined to form mixtures as demonstrated by the presence of additional lines of precipitation in Ouchterlony plates. They also reported that PA was composed of two fractions whose proportions shifted with purification or storage. Stanley and Smith (1961) and Wilkie and Ward (1967) speculated that all three components join in some sort of a loose complex, probably as a result of the chelating action of EF. If the components do exist as a complex, then

only in the presence of 0.1 *M* pyridine solutions, pH 8.0, and not in the presence of a variety of other routinely used buffers (Fish *et al.*, 1968a).

Buzzell (1967) reported that molecular weights of proteins associated with lethal activity formed a series of 8,500, 17,000, 34,000, and 51,000, while we obtained a value of approximately 100,000 for both PA and LF (Fish and Lincoln, 1967; Fish *et al.*, 1968a). In view of the apparent structural and configurational complexity of these molecules, however, it is apparent that much more work is needed.

Toxoiding of the toxin or components has been shown and suggested repeatedly (H. Smith *et al.*, 1954; Beall *et al.*, 1962; Molnar and Altenbern, 1963; Fish *et al.*, 1968a), and this area must be re-evaluated in the light of new definitions (Bonventre *et al.*, 1967a) for toxins and toxoids.

B. Summary and Future Research

Workers are not in full agreement regarding any of the physicochemical properties of the components of toxin, and this area requires more extensive work with purified components. In general, all three components appear to be proteins or lipoproteins that are most stable when stored at 0–4°C and pH 7.4–8.0.

The evidence for molecular heterogeneity is impressive but circumstantial since no knowledge exists as to the molecular configuration of the toxin molecule produced by the organism either *in vivo* or *in vitro*. Consequently, the lack of agreement among workers as to the characteristics of the individual components may be due either to fundamental changes in the structure of the toxin or to artifacts induced by purification procedures.

Undoubtedly, many of the results discussed here will have to be re-evaluated as we learn more of the structure and complexity of this toxin. Much of our present information arose from incidental observations on toxin produced or purified in different ways. With the use of a toxin standard (Haines *et al.*, 1965) and the new techniques for toxin purification now available (Wilkie and Ward, 1967; Fish *et al.*, 1968a), much useful information can be obtained.

V. Synthesis

A. *In Vivo* Toxin

The relationship between the number of bacilli and the units of toxin present in the terminal blood of six host species is summarized in Table II

toxin in the blood of animals dying from anthrax but attribute this to different attachment sites for the antiserum and various components of toxin. The coexistence of antigen and antibody in the blood was noted early in the study of immunology (Wells, 1929). These observations (Ward *et al.*, 1965; Fish and Lincoln, 1968) appear best explained by the observation of Opie (1923) that, in the presence of an excess of either antigen or antibody, the Ag–Ab combination is inhibited and both components remain in the free uncombined state.

One of the most interesting pieces of work, which unfortunately has never been pursued, is the amino acid antimetabolite studies of H. Smith and Tempest (1957) and Tempest and Smith (1957). During the terminal 3 hours of bacteremia, the plasma concentration of 4 (glutamine, glycine, threonine, tryptophan) out of 16 amino acids decreased markedly, while the other 12 increased markedly, except serine which was unchanged. When 57 amino acid analogs were injected, one (8-azaguanine) inhibited both bacterial growth and toxin formation at the same rate, some (ethionine, *p*-fluorophenylalanine, and α-amino-*n*-butyric acid) inhibited bacterial growth and toxin formation at different rates, and some (2-thiouracil and pyridine-3-sulfonic acid) inhibited toxin production without decreasing the growth rate. This indicated that various pyrimidines or nicotinamide might be involved in the synthesis or release of toxin or of one component. The ability to divorce toxin production from bacterial growth should greatly aid studies on toxin synthesis, its control, and exploitation.

B. *In Vitro* Toxin

Initial efforts to demonstrate toxin production were unsuccessful because of its unexpectedly early appearance and rapid destruction (Gladstone, 1948; H. Smith *et al.*, 1956b). Successful production has since been achieved using either bicarbonate in place of serum albumin (Gladstone, 1946), blood aerated with 20% carbon dioxide (Harris-Smith *et al.*, 1958), gelatin in place of serum (Thorne *et al.*, 1960), or a simple salts medium (Haines *et al.*, 1965). Wright *et al.* (1954a) noted the requirement of proline and threonine for "protective antigen" production rather than growth and speculated that its elaboration was associated with a particular type of metabolic activity. Puziss and Wright (1954) noted that omission of isoleucine, leucine, histidine, aspartic acid, glutamic acid, arginine, methionine, proline, tryptophan, or Ca^{2+} from the growth media had no effect on growth of the organism but inhibited "protective antigen" elaboration. Gladstone (1946) reported that bicarbonate was not required for buffering activity and that it could not be replaced as a one-carbon donor by citrate, succinate, fumarate or malate. Puziss and Wright (1954) noted

transient nature of toxin produced *in vitro* (Harris-Smith *et al.*, 1958; H. Smith and Stanley, 1962; Aleksandrov *et al.*, 1964), and (4) instability of *in vivo* toxin after serum is collected from the host (Fish and Lincoln, 1967, 1968).

D. Summary and Future Research

A positive correlation exists *in vivo* between the number of bacilli and units of toxin in the serum or lymph. Toxin production is independent of bacterial growth, and selective utilization of this observation should allow for many future kinetic studies.

Toxin can be produced *in vitro* in a variety of media with carbon dioxide being required for production or release of toxin from the cell. Nutritional requirements for the production of toxin or its components are not well understood.

Toxins produced *in vitro* and *in vivo* are closely related; however, important differences have been noted. The kinetics for the production of each component of this toxin need elucidation, considering not only the *in vivo* and *in vitro* situation but also the bacterial strain and supplementary or growth inhibiting substances. The isolation of strains (1) producing a new toxin component, (2) lacking one of the known components, or (3) with widely different ratios of the components (as compared to the Sterne strain) are attractive possibilities for research and immunization.

Understanding the genetic control for the production of these components will be a challenge for many years. The interesting observation that free circulating antigen and antibody coexist in the blood of immunized hosts at death from anthrax needs to be explained with respect to (1) rates of synthesis of components (or the whole toxin), (2) the kinetics of the Ag–Ab binding, and (3) the binding affinity of the antiserum versus host tissue.

VI. Pathogenesis

A. Anthrax Infection

Anthrax occurs in two forms. The first is a localized or cutaneous infection manifested by an intense, dark, open eschar. This form occurs in only a few species (man, swine, rabbits, horses), and although the eschar is ugly and open, it is surprisingly nonpainful and is readily healed by antibiotics (Cromartie *et al.*, 1947a). The generalized or septicemic form of

VII. Mode of Action

A. General Remarks (Stamatin, 1964a,b; Lincoln *et al.*, 1964a; Nungester, 1967)

Sterne (1961) noted, "Perhaps we know more about the way anthrax bacillus works than we do about almost any other pathogen of similar invasiveness," and H. Smith and Stoner (1967) concluded, "It seems generally accepted now that death in anthrax is due to toxin." The histopathology of the bacillary disease and of the toxemia from sterile toxin are quite dissimilar, yet the pathophysiology is remarkably similar. In some cases, there have been tendencies to (1) extrapolate from observations on the bacillary disease to the action of the toxin, (2) work only with toxin or a single host and extend conclusions generally, and (3) ignore the fact that what is causal in one host is not causal in another. Each group working in this field has reached a different conclusion as to the primary cause of death. The principal theories as to cause of death are blockage of capillaries by bacteria (Vaughan and Novy, 1902), cardiovascular failure (Middleton and Standen, 1961), effect on the reticuloendothelial system (Albrink, 1961), oxygen depletion (Nordberg *et al.*, 1961), "secondary" shock (H. Smith *et al.*, 1955a), increased vascular permeability (H. Smith and Stoner, 1967), and respiratory failure of central nervous system origin (Vick *et al.*, 1968). In this section, we first will review the reported experimental data on the effect of toxin on the host and then follow with a critical discussion of these data as they affect the theories of the cause of death.

Death from anthrax may be sudden and unexpected, and essentially all mammals, as well as some birds and reptiles, are susceptible. Consequently, specific signs or symptoms of internal or generalized anthrax (i.e., temperature and hematological changes, depression, paralysis, or changes in heart rates or patterns) do not exist. Because recognition of anthrax is based principally on the presence of gram-positive bacilli, and septicemia may not occur in some species, many anthrax deaths go undetected (Dordevic, 1951; Sterne, 1959). Signs in the dead host are more characteristic—rigor mortis is delayed, the blood appears dark red and unoxygenated, and bloating is rapid.

A consideration of either the principal hypotheses on mode of action or the diverse pathology and physiology reported for this disease will indicate that it may attack many organs and physiological systems or functions. However, we find only one universal symptom, and it is one that occurs in both the bacterial and toxigenic disease; namely, respiratory failure. But respiratory failure may occur as a result of central nervous

arterial and venous blood, respectively (Eckert and Bonventre, 1963; Klein *et al.*, 1966). In all other diseases except anthrax—and including death by drowning or asphyxiation—(Nordberg *et al.*, 1961), the oxygen level at death remains above 5%. The low blood oxygen level was not due to destruction of erythrocytes or to a decreased oxygen-binding capacity of hemoglobin, because gentle agitation changed the color from the dark red of slow-clotting blood characteristic of anthrax to the bright red of normal oxygenated arterial blood (Eckert and Bonventre, 1963).

Following toxin challenge in rats, a marked hyperglycemia occurs (Eckert and Bonventre, 1963), and a hypoglycemia develops as the toxemia progresses, while liver and muscle glycogen are utilized to maintain "normal" serum glucose levels (Fish *et al.*, 1968b). Serum lactate was elevated about 3.5 times at death (Gray and Archer, 1967). In rabbits injected with sublethal doses of toxin, a marked hyperglycemia occurred, which could be mediated by epinephrine and blocked by ergotamine, but no consistent hypoglycemia developed (Slein and Logan, 1960). The hyperglycemia required both PA and EF (later shown to contain LF) (Beall *et al.*, 1962) and was prevented by antiserum. Blood levels of K^+, Cl^-, and PO_4^{-3} rose, while Ca^{2+}, Na^+, carbon dioxide, and pH decreased (Klein *et al.*, 1966). Blood cholinesterase in rabbits increased, then gradually decreased (Klein *et al.*, 1966). Survivors showed inhibition equal to those dying, and the effect of cholinesterase inhibition may simply accentuate signs of toxemia, such as spastic paralysis.

Slein and Logan (1960), using only one 1-kg rabbit per treatment, injected nonlethal doses of toxin IV. They reported no change in serum glycoprotein levels, but increases in serum aldolase, phosphoglucose isomerase, glutamic/oxaloacetic acid transaminase, amylase, alkaline phosphatase, and cholesterol. Antiserum injected with the toxin diminished but did not prevent these changes. Hyperphosphatasemia occurred after injection of PA or PA plus EF, but not EF alone, and this response was not inhibited by antiserum or bilateral nephrectomy. The rabbit is a particularly variable animal as regards response to weal formation (Belton and Henderson, 1956) and demonstrates no dose response to anthrax spore challenge (Walker *et al.*, 1967). Consequently, more observations must be made on this species than are required on other species to gain equal precision and confidence.

3. Blood Cellular Elements

Toxin resulted in a marked shift to the left of blood cellular elements, and the increase in blood leukocytes was correlated with an increase in polymorphonuclear leukocytes (Klein *et al.*, 1966; Fish *et al.*, 1968b). Eosinophiles are not a feature of anthrax intoxication; however, nu-

some associated cycling of respiration occurred with the rat (Fish *et al.*, 1968b). Subcortical changes in electrical activity occurred simultaneously with the surface cortical changes (Vick *et al.*, 1968). All of these changes were prevented by specific antiserum (Klein *et al.*, 1967; Fish *et al.*, 1968b; Vick *et al.*, 1968). Initial changes in electroencephalogram readings were caused by PA but not LF alone (Vick *et al.*, 1968). In the monkey, changes in the respiration rate were associated with repeated and irregular discharges over the phrenic nerve, which did not synchronize with the inspiration phase of respiration. At all times, from challenge until death, the phrenic nerve remained capable of transmitting an electrical discharge, and stimulation of the cut end of the phrenic nerve elicited a hyperactive response in the diaphragm. Thus, there was no indication of a block in the neuromuscular transmission; rather, the brain was depressed and no longer capable of initiating an electrical discharge (Vick *et al.*, 1968).

Whereas 10,000 rat units of toxin injected IV in monkeys caused death at 28–34 hours, injection of 1000 units directly into the cerebrospinal fluid produced death in 6–10 minutes (Remmele *et al.*, 1968). An immediate tetanic paralysis and cessation of respiration followed injection of toxin, and the central venous and arterial pressures rose sharply. In spite of a heart rate that initially decreased but immediately returned to normal, aortic blood flow dropped precipitously and remained low until death. Electrocardiogram changes were consistent with myocardial hypoxia. Intensive muscle fasciculation developed, and blood pressure and heart rate decreased, progressing to prolonged hypotension, loss of rigidity, and death. Introduction of toxin into the cerebrospinal fluid resulted in a great stimulation of central nervous system discharges as indicated by muscle contraction and changes in blood pressure and flow that led rapidly to anoxia and death. In spite of these extreme changes, survival was obtained either by artificial ventilation of only 10 minutes duration or by two injections of the β-adrenergic stimulant isoproterenol at 420 and 650 seconds post challenge.

C. Critical Evaluation of Hypotheses on Cause of Death

We feel that the primary cause of death must be identical in all hosts whether one considers the bacillary disease or the injection of sterile toxin. For this reason, attributing death to blocking of the capillaries by bacilli (Vaughan and Novy, 1902) may be rejected outright because no bacilli or particulate material is injected with the toxin, yet, the general physiological signs of disease are the same (H. Smith and Keppie, 1955; Klein *et al.*, 1966, 1968). Six other hypotheses are considered below.

in the brain. However, this and similar work should be pursued with more careful attention paid to the use of controls, other host species, and recognition that the response of nervous tissue to toxins can be very selective. For example, botulinum neurotoxin does not affect oxygen consumption of brain slices nor does it show any significant central nervous system effects *in vivo* (Stevenson, 1962), whereas cobra venom, which can affect the central nervous system, modifies oxygen consumption of brain slices *in vitro* (Quastel, 1957). Thus, it is possible that the 21% increase in oxygen consumption of brain brei noted by Gray and Archer (1967) is a modification of cellular respiration directly attributable to toxin.

6. Respiratory Failure of Central Nervous System Origin

This model was proposed by Lincoln *et al.* (1964a), criticized by Smith and Stoner (1967), and supported by Vick *et al.* (1968), Remmele *et al.* (1968), Klein *et al.* (1968), and Fish *et al.* (1968b). Respiratory failure is a sign observed for all hosts and frequently is so acute as to cause death which is repeatedly described as "sudden" or "unexpected." The wide range of changes in physiology, enzymatic activity, blood chemistry, and blood cellular elements are nonspecific and believed to be consistent with central nervous system interaction. We (Vick *et al.*, 1968) have demonstrated depression of electroencephalogram tracings and a lack of conductance over the phrenic nerve without detectable effect on neuromuscular and diaphragm function. Lack of demonstrable histological changes in neural elements or in other organs (Bonventre *et al.*, 1967b; Vick *et al.*, 1968) does not necessarily imply lack of physiological involvement. We (Remmele *et al.*, 1968) have shown that survival occurs if respiratory failure is overcome by either forced ventilation or isoproterenol. Specific antiserum prevents depression of electroencephalogram activity and symptoms of respiratory distress.

D. Summary of Current Status on Mode of Action of Toxin

Most of the hypotheses presented to explain the mode of action of toxin deal with secondary effects, and while these may cause death, they are not the primary "mode of action" of the toxin. Gross pulmonary edema appears to be restricted to toxin-challenged Fischer 344 rats; it is not marked in any of the other species in which anthrax has been described or the other six species challenged with toxin. Pulmonary edema may be caused directly by central nervous system injury (Reynolds, 1963), and in fact, Reynolds' description of rats dying from pulmonary edema resulting from electrolytically induced lesions of the hypothalmus is remarkably

however, because the methods used to obtain the components varied with each group, the optimum PA:LF ratio has not been established. As greater amounts of PA are added to a constant amount of LF, the time to death reaches a minimum, then increases to survival; however, as more LF is added to a constant amount of PA, the time to death continues to decrease (Molnar and Altenbern, 1963; Fish *et al.*, 1968a). Injection of toxin via the carotid artery or via the tail or femoral vein did not change the time to death of rats (Bonventre *et al.*, 1967b). Regardless of dose, 55 minutes is the minimum time to death for rats challenged with *in vitro* toxin (Haines *et al.*, 1965; Beall and Dalldorf, 1966; Fish and Lincoln, 1968); however, toxin produced *in vivo* may kill in a much shorter time period (Fish and Lincoln, 1968). Generally, the more resistant a host is to the establishment of bacillary infection, the more susceptible it is to toxin (Table III) (Klein *et al.*, 1963a; Lincoln *et al.*, 1967).

Toxin action is dependent upon (1) the kinetics of attachment to the host tissue, (2) the amount and configuration of each component, (3) the host and any stress conditions, and (4) pharmacological treatment of the host.

G. Treatment

Medical treatment of anthrax, except Russian use of antiserum (Mashkov, 1958), ignores the toxin and attempts to remove the bacillus and perhaps give symptomatic support. It is notable, too, that Russian recommendations specifically exclude symptomatic cardiac support (Yablokov, 1950). Prior to antibiotics, "Hyperimmune serum and neosalvarsan were the most rational items of clinical use" (Sterne, 1959), and the case re-

TABLE III

Relationship between the Number of Anthrax Bacilli Required to Establish Infection and the Units of Toxin Required to Cause Death[a]

Host	Spores required to establish infection	Dose required to cause death (units/kg)	Time to death (hours)
Mouse	5	1000	24
Guinea pig	50	1125	24
Rabbit	5000	2500	72
Rhesus monkey	3000	2500	28
Chimpanzee	—[b]	4000	60
Rat, Fischer 344	0.7×10^6	15	2
Rat, NIH Black	1.5×10^6	280	20
Dog, beagle	5×10^7	60	20

[a] From Lincoln *et al.* (1967).

[b] No data; considered susceptible.

H. Miscellaneous Responses to Toxin

Toxin (1) is virulence-enhancing, (2) inhibits phagocytosis of *B. anthracis* cells by guinea pig polymorphonuclear leukocytes, (3) is anticomplementary, and (4) inhibits the action of anthracidal substances in normal serum and in extracts of leukocytes (H. Smith and Gallop, 1956; H. Smith *et al.*, 1956a; Kashiba *et al.*, 1959; Lincoln *et al.*, 1964a). Toxin (aggressin) also may play a role in invasiveness (Bail, 1904; H. Smith, 1958), although this conclusion is based on *in vitro* observations with relatively high concentrations of toxin. The latter may not reflect toxin action as an aggressin *in vivo*, where few bacterial cells occur and toxin must be dilute, localized, and follow rather than precede cell growth. In addition to the capsule and toxin, other chemical substances may act as aggressins (Sterne, 1948a,b; Young and Zelle, 1946; Ivanovics, 1964).

Toxin containing an unknown amount of guinea pig serum produced a granuloma in rats which had a high fluid content and appeared histologically as a very loose cell structure. Antiserum prevented formation of the granuloma. When this toxin was instilled into the eye of the rabbit, a nonspecific vasodilation but not chemosis was observed. This toxin neither promoted nor inhibited migration of leukocytes from chicken blood clots (Desaulles *et al.*, 1956).

There is no visible cytopathological effect of toxin on three tissue cell lines (Bonventre, 1965). However, crude toxin liberated β-glucuronidase but not acid phosphatase from large granules derived from rabbit livers. When anthrax bacilli were grown in human embryo cell cultures (Ginsburg and Maslova, 1963; Ginsburg and Fedotova, 1963), the virulent bacilli grew attached to the tissue cell and caused a cytopathogenic effect, whereas hypovirulent bacilli grew free in the medium and caused no cytopathogenic effect. Although toxin was not demonstrated, this response was attributed to the "toxic factor of virulence" (toxin). Crude toxin did not decrease turbidity of suspensions of lysosomes (Bernheimer and Schwartz, 1964). Kashiba *et al.* (1959) noted that the terminal serum of guinea pigs dying of anthrax produced changes in the phagocytic and anthracidal activity of white blood cells from ten host species. Those species whose leukocytes were most readily prevented from phagocytizing killed staphylococcal cells were the guinea pig, cow, man, rabbit, sheep, horse, and mouse, whereas the rat, dog, and swine were resistant or refractive to the serum. Although unable to repeat these observations, Lincoln *et al.* (1967) noted that their data showed an inverse correlation between maximum dilution of terminal serum to inhibit phagocytosis and susceptibility of the host to establishment of anthrax.

EF is reported as virulence-enhancing, antiphagocytic, and anthracidal

which protects against challenge. Not only has this connotation proved erroneous (Stanley and Smith, 1963; Mahlandt *et al.*, 1966), but the name itself also had prior use both for the complete toxin (Gladstone, 1946) and for anthrax lesion extracts and exudates from animals dying of anthrax (Cromartie *et al.*, 1947b).

At least six "protective antigens" have been introduced into the literature: (1) edematous extract, (2) Gladstone's antigen, (3) Boor-Tresselt's antigen, (4) Belton-Strange's antigen, (5) aerobic antigen, and (6) anaerobic antigen. These may be classified as those derived from body fluids (1, 2, 3), that produced in a complex undefined medium (4), and those produced in defined synthetic media (5, 6). In none of the "protective antigens" has the component composition and ratio or molecular state of the components been determined; however, there is little doubt that the antigen is related to the toxin.

A. Edematous Fluid and Tissue Extracts

Bail (1904) demonstrated the efficacy of edematous extracts as a vaccine. Salisbury (1926) immunized range animals with "aggressin" (edematous fluid) and found that 27% of the 2684 unimmunized but only 0.32% of the 10,814 immunized animals died. This antigen immunized rabbits, guinea pigs, mice, hamsters, and sheep against various, mostly low, challenge doses of spores (Cromartie *et al.*, 1947b; Watson *et al.*, 1947).

Although effective, production of this vaccine remained an art (Cromartie *et al.*, 1947b), and therefore, potentially more readily controllable methods were developed.

B. Gladstone Protective Antigen

Gladstone (1946) reported production of a "protective antigen" in static cultures containing the plasma or serum of various species. Its immunizing properties were similar to those of anthrax edematous fluid. Rabbits, monkeys, and sheep were immunized against a spore challenge of about 100 lethal doses. Extended time to death for guinea pigs indicated some immunity, whereas mice were not immunized. Extensive tests conducted with rabbits (Table IV) showed excellent protection if two or more administrations of antigen were given. Passive immunity could be transferred. Later, Gladstone (1948) increased the yield of this antigen about 25 times by growing the culture in cellophane bags continuously perfused with serum and aerated broth. The observations of Gladstone were verified (Heckley *et al.*, 1949) and extended to growth in a synthetic medium containing 20% serum (Wright *et al.*, 1951).

TABLE IV
Resistance to Anthrax Challenge Provided by Immunization with Five Different Antigens

Virulent challenge protocol

Further refinement of the protective antigen was achieved by gradual substitution (1) among the medium ingredients, ultimately resulting in a defined medium with no added protein, (2) in strains, (3) in fermentation procedures, and (4) in processing procedures. Each was a minor change, but overall, this development of an effective vaccine was "undoubtedly among the most interesting and important achievements of experimental microbiology in the present century" (Ginsburg, 1964).

C. Boor-Tresselt Antigen

Boor and Tresselt (Boor, 1954; Boor and Tresselt, 1954a,b; Tresselt and Boor, 1954) produced and tested the first practical vaccine (Table IV). The final vaccine was produced with the CD-2 strain (intermediate virulence) and a medium consisting of serum albumin (homologous for the species of animal to be immunized), a fractionated yeast extract, phosphate buffer, and bicarbonate. Fermentation was static. Processing consisted of centrifugation and Seitz filtration followed by precipitation (with either alcohol or ammonium sulfate), dialysis, and lyophilization.

D. Belton-Strange Antigen

A nonprotein complex medium containing activated charcoal was used to produce the Belton-Strange antigen (Belton and Strange, 1954). This antigen was grown in static culture, sterilized by filtration through sintered glass, and then concentrated either by (1) lyophilization or (2) alum precipitation. This vaccine gave good protection (Table IV), and although the circulating antibody level was low, a marked amnestic response of "at least" 64-fold occurred following challenge. Simpson (1966) used this antigen in cattle in North Africa and found that immunity, as assayed by Ouchterlony agar gel diffusion, was too variable to evaluate.

E. Aerobic Antigen of Wright and Colleagues

Initially, this immunogen was produced in the chemically defined medium of Brewer *et al.* (1946) at $\frac{1}{5}$ concentration and with 0.03 *M* sodium bicarbonate added. Aerobic (thin layer, static culture) growth (Wright *et al.*, 1954a,b; Puziss and Wright, 1954) distinguishes this immunogen from that produced under anaerobic conditions (described in Section F).

The aerobic "protective antigen" was developed in five media (528, 555, 599, 687, and 968) utilizing nonproteolytic (NP) and NP-nonencapsulated (R) strains selected from seven stocks (Vollum, V770, 116, 107, 108, 1062, and 1133). In conducting the research on this immunogen,

injection of PA increased susceptibility by about 100-fold (Keppie *et al.*, 1953; Lincoln *et al.*, 1964a).

It does not appear that the aerobic protective antigen produced from the seven strains or their variants in the five growth media differed among themselves, and there was no evidence of antigenic heterogeneity based on challenge of guinea pigs.

Two antigens prepared with different strains and media were used by Schlingman *et al.* (1956) for yearling cattle, sheep, and hogs. The statistical design of the cattle experiment was confounded so that one lot of antigen appeared to immunize, the other not to immunize, against oral challenge. Sheep were partially immunized against a 20,000-spore dose. Swine tests were inconclusive because even controls could not be infected.

An antigen produced with a different medium and strain combination was used by Jackson *et al.* (1957) with cattle challenged orally (Table IV). Partial immunity was obtained, but its development required two injections of antigen and a 30-day interval prior to challenge. Persistence of immunity was short. It is of interest that all six cattle immunized with live vaccine survived challenge at 3 months.

Brachman *et al.* (1960, 1962) used a similar antigen in tests on humans employed in four goat-hair processing mills. These mills employed 1249 people, of which 379 were vaccinated with three injections of antigen. Twenty-six cases of anthrax occurred; one was in vaccinated and two in incompletely vaccinated individuals. Statistical evaluation based on person-months of exposure (excluding the two incompletely vaccinated cases) indicated the antigen was 92.5% effective. However, the confidence limits (65-95%) were very questionable because of (1) several assumptions, (2) few anthrax cases, and (3) changes from the original experimental design. It is true that the Manchester epidemic (9 cases) was stopped (Brachman *et al.*, 1960) simultaneously with the immunization of all employees; however, the goat-hair lot believed to be the source of infection also was removed. As noted by Zhdanov (1961), such results cannot be considered conclusive because of nonuniform exposure.

F. Anaerobic Antigen

Stirred anaerobic cultures (Wright and Puziss, 1957; Puziss and Wright, 1959; Wright *et al.*, 1962; Puziss *et al.*, 1963) also produce an immunogen (Table IV). This process uses a vegetative inoculum of the V770-NP1-R strain, and harvest is based on glucose utilization. Antigen is precipitated with preformed aluminum hydroxide gel (the alum process

TABLE V
MEASURE OF IMMUNITY OF GUINEA PIGS BY THREE ANTHRAX ANTIGENS WITH AND WITHOUT BOOSTER INJECTIONS[a]

Measures	Antigen type					
	Aerobic		Anaerobic		Belton-Strange	
	Primary	Booster	Primary	Booster	Primary	Booster
Mean immunity index[b] ± σ	1.3 ± 1.4	5.2 ± 0.70	3.3 ± 0.41	3.8 ± 0.47	2.4 ± 0.25	4.8 ± 0.52
Coefficient variation (%)	107	14	12	12	10	11
Positive titers						
Percentage	17	50	42	100	25	100
Range	1:2 - 1:4	<1:2 - 1:16	< 1:2 - 1:8	1:4 - 1:16	< 1:2 - 1:8	1:8 - 1:128
Mean	<1:2 ± 1:13	1:4 ± 1:15	1:20 ± 1:08	1:83 ± 1:08	1:13 ± 1:06	1:45 ± 1:10
Coefficient variation (%)	7	38	41	11	44	27

[a] From Klein *et al.* (1963b).
[b] Immunity index is the $\log_{10}$ increase in dose required to cause the immunized host to respond (die) at the same time as the control.

and (6) the number of bacilli and units of toxin per milliliter in the blood decreased as immunity increased and were therefore inversely related to host resistance. Essentially, only LF-immunized guinea pigs developed antigen-antibody precipitin lines on Ouchterlony plates, while rat sera were all negative. Serum antibody binding titers were not conclusive. Fish *et al.* (1968a) showed that greatly increased immunization was produced by equivalent amounts of purified PA compared to crude culture filtrates.

J. Antigen-Live Vaccine Interaction

Consistent, tremendously increased resistance has been noted following booster with live vaccine (Table VI) (Klein *et al.*, 1962b, 1963b). The injection of additional antigen or of live vaccine results in a 2.5 or 5.0 $\log_{10}$ increase, respectively, in resistance to challenge. This suggests that the present antigen and/or procedure is incomplete regarding development of full immunity in experimental animals and presumably in man.

When immunity is developed with the PA_5 (5 injections of PA) plus live vaccine, guinea pigs were not killed by doses of 1×10^9 spores (Klein *et al.*, 1962b) or rhesus monkeys by 1×10^{10} spores (Lincoln *et al.*, 1964a).

K. Variation in Immunity Protocols

The rabbit, which was used by essentially all early workers for immunization studies, does not demonstrate a dose-response curve through an 8-log spore dose (Walker *et al.*, 1967) and is heterogeneous to edema test

TABLE VI
Response of Guinea Pigs Immunized by Different Protocols Using Belton-Strange or 30R Live Vaccine[a]

Immunization protocol[b]	Day of injection of antigen			Mean time to death (hours)	Immunity index[c]
	PA (0.1 ml)	PA (1.0 ml)	10^7 spores		
Control	0	0	0	22	0
PA_5	1,3,5,9,11	0	0	32	3.2
PA_5+PA_1	1,3,5,9,11	22	0	48	5.8
PA_5+LV_1	1,3,5,9,11	0	22	91	8.2
PA_1	0	1	0	32	3.2
LV_1	0	0	1	23	0

[a] From Klein *et al.* (1962a).
[b] The subscript number refers to the number of injections of the indicated component.
[c] Immunity index is the $\log_{10}$ increase in dose required to cause the immunized host to respond (die) at the same time as the control.

L. Collection of Multiple Data

It is unfortunate that more data are not obtained from each experiment as this is largely a matter of planning and concentration of effort. The need to be highly efficient seems obvious when certain species are used; i.e., chimpanzee (critically scarce and verging on extinction) or horses or cattle (unusually expensive). Even with the small, easily raised species, man has a moral obligation while experimenting to obtain as humanely as possible as much useful information as possible. We suggest that the customary observation of whether an animal is alive or not at the beginning and end of the working day is not only inefficient but also scientifically questionable. Continuous observations can be obtained with time-lapse photography, and temperature changes and other physiological parameters can be monitored electronically. The problem of determining the optimum concentration of antigen and the best protocol for immunization is difficult, and Stanley and Smith (1963) and H. Smith (1964) have called it unsolvable because of the large number of animals needed. However, Mahlandt *et al.* (1966), state that the problem can be solved by using (1) modern statistical design, such as the factorial experiment, (2) quantitative methods, such as the immunity index, and (3) multiple criteria of evaluation, such as five criteria rather than one to evaluate immunity.

M. Summary and Future Research

Six types of "protective antigen" vaccine have been developed; however, only "chemical" antigens (those produced in media lacking serum or tissue extracts) have been developed extensively. While the chemical antigens undoubtedly increase resistance to anthrax challenge, it remains to be seen if their use can control anthrax in a high-risk area. Immunity developed by the chemical vaccine does not equal that obtained with either the living vaccine (Sterne strain) or protective antigen plus live vaccine, and antibody titer is low and transitory. It is quite likely that large amounts of antigen, more frequent boosters, and the addition of more components or live, attenuated spores would greatly enhance the potency of the vaccine.

Brachman (1966) reviewed industrial inhalation anthrax, presented data showing the cases of anthrax for the past 52 years, noted that anthrax is becoming extinct in the United States; he attributed this to the introduction of the chemical vaccine in 1955. However, the long-term trend has not changed slope, and it is possible that other factors (e.g., better ventilation, dust control, cleaner sources of wool or hair) are more important than immunization (Gold, 1967).

suppose that the toxin is somehow useful in the organism. Some knowledge concerning the kinetics of toxin production and its function in the cell is desperately needed. We suggest that this information might form a basis for understanding the larger problems of evolution among species and the development of pathogenicity or saprophyticity. Why is a complex toxin evolutionarily selective; why not a simple toxin or a still more complex one? Perhaps the molecular heterogeneity of the toxin molecule gives it some selective advantage. It is intriguing that, in the sporogenic *Bacillus-Clostridium* complex, many species produce either toxins, antibiotics, or pigments. Are these apparently different materials related in some fashion? If so, antibiotic production might reflect a mutational event leading to a slightly altered toxin. *Bacillus anthracis* and *B. cereus*, held by some (N. R. Smith *et al.*, 1952) to be the same species, produce toxins giving distinctly different host responses, use of which Bonventre and Eckert (1963) have suggested as a means of species identification.

As a research tool in toxicology, anthrax toxin is not an ideal agent since it causes multiple responses; however, certain unique responses suggest its use until simpler material becomes available. Because it is composed of three components, each of which is nonlethal in purified form, this toxin offers the chance to study certain interactions not possible with other toxic materials. It is one of the few materials of rather large molecular weight that can affect the central nervous system rapidly and should prove useful in studying the "blood-brain" barrier and possibly, when employed in sublethal concentrations, for effecting the passage of other drugs into the central nervous system. A pulmonary edema of controlled time and possibly intensity can be developed in the Fischer 344 rat. This edema probably is of central nervous system origin and makes anthrax toxin a new tool for research in this area. Another phenomenon which warrants investigation is the extraordinarily low level of oxygen in the arterial blood at death. What mechanism operates which results in depletion of essentially all oxygen, whereas with drowning, suffocation, and certain diseases (Nordberg *et al.*, 1961), the oxygen level does not fall below 5–6%? The fact that either time to death or time for the development of edema cannot be reduced below a minimum period, even with increased toxin concentration, indicates that its action is not enzymatic. Whether the epinephrine-like material recently described in culture filtrates (Williams *et al.*, 1967) is related to toxin is not known, since the absence of toxin or components was assumed.

The interaction of this pathogen or its toxin with the host is of particular interest because establishment or treatment of the disease and the terminal toxemia can be studied separately. The inverse relationship between the dose of spores required to establish anthrax and the level of bacilli or

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